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Cleavage of supercoiled double-stranded DNA by several ribosome-inactivating proteins in vitro

Jun Ling, Wang-yi Liu, T.P. Wang*

Shanghai Institute of Biochemistry, Academia Sinica, Shanghai 200031, China

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Abstract

Several ribosome-inactivating proteins (RIPs), such as ricin (including its A-chain), luffin, cinnamomin and camphorin, were found to express enzymatic activity to cleave supercoiled double-stranded DNA. In particular, α-sarcin, a RIP with a novel ribonuclease activity, was first proved to have this activity. They convert supercoiled DNA into a nicked circular conformation at low concentrations and further into a linear form at high concentrations: they have no effect on linear DNA. Although intact type II RIPs exhibited no RNA *N*-glucosidase activity, they were detected to cleave supercoiled DNA. Even if ricin A-chain was treated by boiling, its activity on supercoiled DNA was largely retained.

Key words: Ribosome-inactivating protein (RIP); Ribosomal RNA; Supercoiled double-stranded DNA; RNA *N*-glycosidase

1. Introduction

Ribosome-inactivating proteins (RIPs) are a group of cytotoxins which potently inhibit eukaryotic protein synthesis by catalytically and irreversibly inactivating the 60 S ribosomal subunit [1]. Apart from a few isolated from fungi and bacteria, most RIPs are widely distributed in higher plants. Based on primary sequence, RIPs are classified into two major types. Type I consists of a single chain with a molecular weight between 25,000–30,000 Da, while type II, with a molecular weight around 60,000 Da, usually consists of two chains (A and B) connected by a disulfide bond. The B-chain binds to the galactose-containing receptor on cell surfaces and facilitates the entry of the A-chain into the cytoplasm where it inactivates the ribosome. According to the molecular mechanism of action, type I RIPs and the A-chains of type II RIPs act as a specific RNA *N*-glycosidase, catalytically cleaving the C–N glycosidic bond of the adenosine residue at 4324 in rat 28 S rRNA, thereby impairing the ability of elongation factor 2 to bind to the eukaryotic 60 S ribosomal subunit [2]. The other enzymatic activity of RIPs is a novel ribonuclease possessed only by α-sarcin hitherto investigated [3]. α-Sarcin is capable of selectively hydrolyzing the single phosphodiester bond on the 3′ site of the guanosine at position 4325 in rat 28 S rRNA.

Besides these activities of RIPs on ribosomal RNA, trichosanthin (type I RIP) was recently reported to have an enzymatic activity which cleaved the supercoiled double-stranded DNA to produce nicked circular and linear DNA [4]. Whether this enzymatic activity is a common characteristic among RIPs is still in question. Clarity of this problem may be of benefit to our understanding of the intrinsic effectiveness of RIPs in eukaryotic cells in vivo. In this communication, we examined several other RIPs, including type II and type I RIPs as well as α-sarcin, and found that all RIPs examined expressed enzymatic activity on supercoiled double-stranded DNA.

2. Materials and methods

2.1. Materials

Ricin was purchased from Sigma Chemical Co., USA. pGEM-4Z, pBR322, pSP70 plasmids and λ DNA were products of Promega Corp., USA. α-Sarcin was a generous gift from Prof. Dr. Norbert Ulbrich (Deutsches Rheuma-Forschungs-Zentrum, Berlin, Germany). Luffin was kindly donated by Dr. Zu-Chuan Zhang of this institute. Cinnamomin and camphorin were isolated and characterized in our laboratory. A-chains, the catalytic chains of ricin and cinnamomin, were purified by the method of Olsnes [5]. Other reagents were all of analytical grade.

2.2. Preparation of supercoiled DNA

Escherichia coli DH 5α transformed with plasmids was cultured in LB medium at 37°C when the culture reached the exponential growth phase, total plasmid DNA was purified by the alkali lysis procedure. The supercoiled DNA was obtained through centrifugation to equilibrium in cesium chloride-ethidium bromide gradients [6].

2.3. Cleavage of supercoiled DNA with RIPs

1 μg of supercoiled DNA (pGEM-4Z) was incubated with serial amounts of RIPs in a final volume of 20 μl containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, pH 8.0, at room temperature for 1 h. At the end of reaction, 10 μl of loading solution (30% Ficoll, 200 mM EDTA, 0.25% Bromophenol blue and 0.25% xylene cyanol FF) were added. Electrophoresis was carried out under non-denaturing condition in 0.5 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA) in a 1% agarose gel. DNA bands were visualized by staining with ethidium bromide.

Incubation of linear DNA (λ DNA and *Hin*dIII-linearized pGEM-4Z DNA) by RIPs was undertaken as described above.

2.4. Preparation of ribosomes and treatment with RIPs

Rat liver ribosomes were prepared by the method of Wettstein et al. [7]. Modification of ribosomes with RNA *N*-glycosidase, extraction of

*Corresponding author. Fax: (86) (21) 433 8357.

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RNAs and electrophoretic analysis were done according to the procedure reported by Endo et al. [8]. Activity of α-sarcin was assayed as described by Stephen and Bodley [9].

3. Results and discussion

3.1. Cleavage of supercopiled double-stranded DNA by several RIPs

Ricin, one of the most intensively studied RIPs, was the first to be selected for the test. When pGEM-4Z supercoiled DNA was incubated with various amounts of ricin A-chain it was found that the extent of supercoiling was obviously altered. The DNA was first nicked to give a nicked circular form, which moved significantly slower than the supercoiled DNA through the agarose gel (top band in each lane, Fig. 1). When the ricin A-chain concentration was increased up to 3 μg, the linear form of DNA emerged (second band from top in Fig. 1), which migrated faster than the nicked circular form but slower than the supercoiled DNA. Similar effects were also observed with pBR322 and pSP70 supercoiled DNA as the substrate (data not shown). As shown in Fig. 1, the linear form of DNA appeared at 3 μg of ricin A-chain, whereas the linear form was not generated until 5 μg of trichosanthin was used, as reported by Li et al. [4], indicating that ricin A-chain is more active than trichosanthin in cleaving supercoiled DNA. Cinnamomin is a type II RIP, the activity of which is also embeded in its A-chain. Treatment of supercoiled pGEM-4Z DNA with cinnamomin A-chain yielded similar results (Fig. 1), but its activity was slightly higher than that of ricin A-chain.

To provide more evidence, the activities of luffin and camphorin (two type I RIPs) were additionally taken into consideration. After incubation of pGEM-4Z supercoiled DNA with increasing amounts of luffin or camphorin, the supercoiled DNA band in the agarose gel became gradually fainter, whilst nicked and linear bands began to appear. As shown in Fig. 2, luffin or camphorin expressed such high activities that the nicked circular form of DNA was completely converted into the linear form with 5 μg of luffin, or digested into two fragments by 5 μg of camphorin. These results may shed light on the elucidation of the molecular mechanism involved in this process.

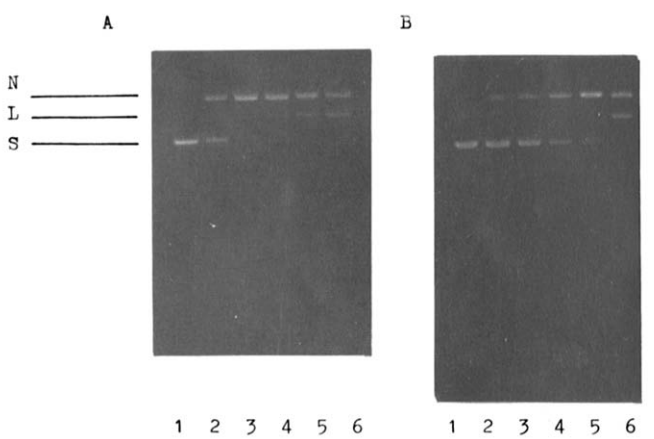

Fig. 1. The enzymatic activity of the A-chains of type II RIPs on supercoiled double-stranded pGEM-4Z DNA. Lanes 1–6 represent 1 μg of pGEM-4Z DNA incubated with 0, 0.1, 0.5, 1, 3, and 5 μg of RIPs, respectively. (A) Ricin A-chain; (B) cinnamomin A-chain. N, nicked circular DNA; L, linear DNA; and S, supercoiled DNA.

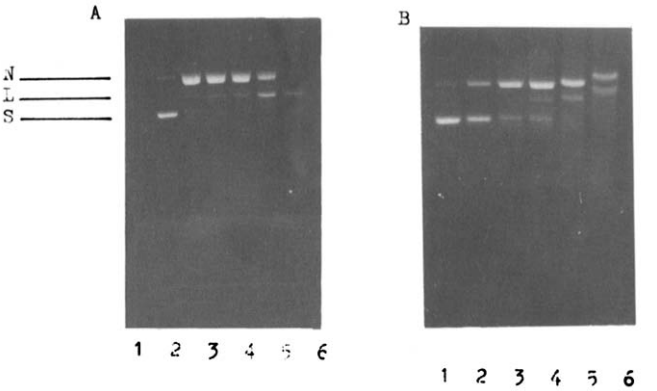

Fig. 2. Cleavage of supercoiled pGEM-4Z DNA by type I RIPs. Lanes 1–6 denote 1 μg of pGEM-4Z DNA incubated with 0, 0.1, 0.5, 1, 3, and 5 μg of RIPs. (A) Luffin; (B) camphorin.

All the above results were obtained with RIPs bearing specific RNA *N*-glycosidase. A wealth of research data demonstrates that RIPs functioning as RNA *N*-glycosidases share an approximately similar three-dimensional structure, especially the consensus conformation of an active center [10]. Therefore the similarity of their activities on supercoiled DNA may be a reflection of the intrinsic identity of their quaternary structure.

α-Sarcin, another kind of RIP, exhibits a novel ribonuclease activity entirely different from RNA *N*-glycosidase. It is worthwhile examining whether α-sarcin is capable of cleaving supercoiled DNA. As displayed in Fig. 3, it was ascertained that α-sarcin exhibited an apparent activity on supercoiled DNA in a fashion similar to that of RNA *N*-glycosidase, although it had a relatively lower

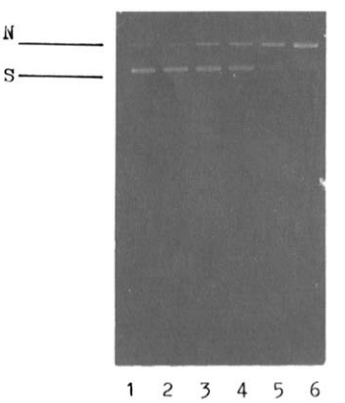

Fig. 3. The effect of α-sarcin on supercoiled pGEM-4Z DNA. Lanes 1–6 indicate 1 μg of pGEM-4Z DNA incubated with 0, 0.1, 0.5, 1, 3, and 5 μg of α-sarcin, respectively.

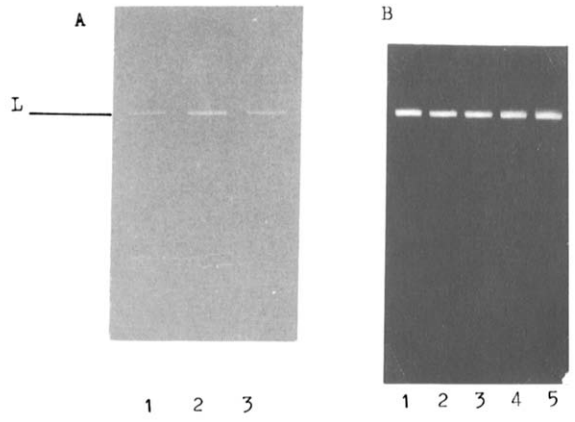

Fig. 4. The effect of RIPs on linear, double-stranded DNA. (A) 1 μg of λ DNA was incubated with 5 μg (lane 2) or 10 μg (lane 3) of ricin A-chain. Lane 1 is the control (without ricin A-chain). (B) Lanes 2–5 represent 1 μg of *Hin*dIII-linearized pGEM-4Z DNA treated with 5 μg of ricin, luffin, cinnamomin or camphorin, respectively. Lane 1 is the control (without any RIPs).

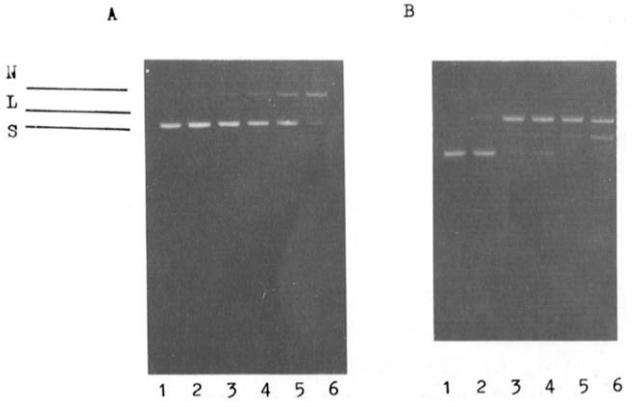

Fig. 5. The enzymatic activities exhibited by intact type II RIPs on supercoiled DNA. Lanes 1–6 represent 1 μg pGEM-4Z DNA incubated with 0, 0.1, 0.5, 1, 3, and 5 μg of RIPs, respectively. (A) Ricin; (B) cinnamomin.

activity. This result implied that a consensus conformation exists between α-sarcin and RNA *N*-glycosidase for their recognition of supercoiled DNA. This presumption is in agreement with the fact that the cleavage sites of α-sarcin and RNA *N*-glycosidase are both located in a strongly conserved purine-rich, single-stranded loop of 14 nucleotides, which is termed the R/S domain [2]. Reinforced by the result from α-sarcin, it can be concluded that enzymatic activity on supercoiled double-stranded DNA is a common characteristic pertaining to RIPs.

As indicated above, there must be a topographic prerequisite for DNA to interact with RIPs. When linear double-stranded λ DNA and *Hin*dIII-linearized pGEM-4Z DNA were used as the substrates, no DNase-like activities were detected (Fig. 4), consistent with the result from trichosanthin. This result demonstrated that supercoiled double-stranded or covalently closed circular DNA was of preference for recognition by RIPs. The fact that RIPs have no activity on linear DNA excludes the possibility that contaminating traces of DNase were present in the RIP preparations.

3.2. *Relationship between activities of RIPs on ribosomal RNA and on supercoiled DNA*

It has been known that intact type II RIPs generally exhibit no activity on ribosomal RNA, e.g. ricin: the two chains must be separated by reduction of the disulfide bond before reaction with ribosome. Actually the RNA *N*-glycosidase activity is bound with its A-chain. In our experiment, however, intact ricin was found to show an unambiguous activity on supercoiled pGEM-4Z DNA. A similar effect occurred with intact cinnamomin, a type II RIP (Fig. 5). By comparing Fig. 5 with Fig. 1 it is clear that the activities of these two intact RIPs are lower than those of their A-chains, demonstrating that there is still some kind of relationship between RNA *N*-glycosidase

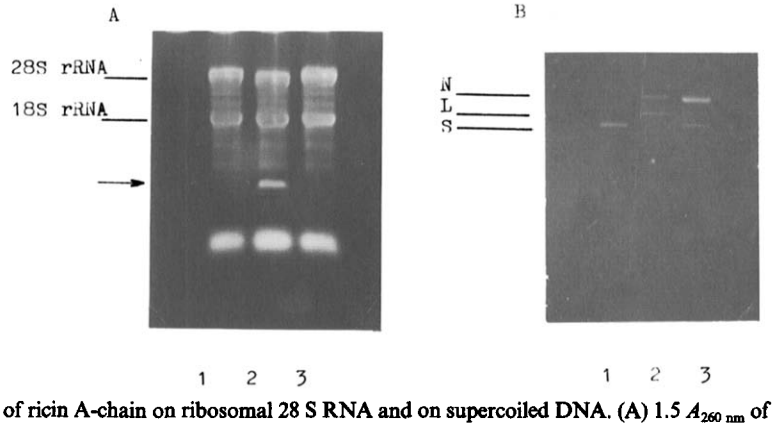

Fig. 6. Comparison of activities of ricin A-chain on ribosomal 28 S RNA and on supercoiled DNA. (A) 1.5 $A_{260\ nm}$ of ribosomes were incubated with 5 ng of ricin A-chain. Following the reaction, rRNA was extracted and treated with aniline, and fractionated on a 1.8% agarose/formamide gel. Lane 1, untreated with ricin A-chain; lane 2, treated with ricin A-chain; lane 3, treated with ricin A-chain inactivated by boiling for 10 min. The arrow indicates the specific R-fragment. (B) 1 μg of supercoiled pGEM-4Z DNA was incubated with 5 μg of ricin A-chain. Lane 1, untreated with ricin A-chain; lane 2, treated with ricin A-chain; lane 3, incubated with heat-inactivated ricin A-chain.

and the supercoil-cleaving activity. These results suggested that RNA *N*-glycosidase and supercoil-cleaving acitivity might be implemented by diverged domains of the RIP molecule. It may be possible that exclusion of the B-chain favours the folding of the A-chain into a preferable conformation for the recognition of supercoiled DNA.

More convincingly, when ricin A-chain was denatured by boiling for 10 min, its RNA *N*-glycosidase activity was entirely inactivated. However, the activity of cleaving supercoiled DNA was still detected, although it was decreased with respect to the non-denatured ricin A-chain (Fig. 6). This result provides further support for the idea that these two kinds of enzymatic properties embedded in the one RIP molecule may not be closely related. Information from the crystallographic structure of RIP, peculiarly concerning its differential domains responsible for recognition and activity [11,12], may offer invaluable clues to finally resolve this problem.

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